**Editorial comments:**

The manuscript has been modified and the updated manuscript, **57900\_R0.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please use 12 pt font and single-spaced text throughout the manuscript.

3. Please ensure that the references appear as the following:

Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi: DOI (YEAR).

For more than 6 authors, list only the first author then et al.

4. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc., and h, min, s for time units.

5. Please use a single space between numerical values and their units.

6. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

7. Figure 1: Please provide a short description of the figure in addition to the title.

8. Figure 2: Please provide a short description of the figure in addition to the title.

9. Figure 3: Please provide a title for the whole figure.

10. For in-text referencing, please remove the brackets before and after the reference numbers and superscript the reference numbers.

11. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

12. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

13. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

14. Step 1): Please write this step in imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”

15. 2)-1: How to fix the fiber? Please explain.

16. 2)-3: How to fix the shutter?

17. 2)-5: Please ensure that all text is written in imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”

18. 2)-8: Please ensure that all text is written in imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”

19. 4)-b.3: Please ensure that all text is written in imperative tense.

20. 4)-b.5: Please ensure that all text is written in imperative tense.

21. 4)-b.7: Please ensure that all text is written in imperative tense.

22. 5)-2: What’s the pore size of the sieve?

23. 5)-3: 100% bleach? Please verify the concentration. Please add it the Material Table.

24. 5)-5: How to select? How many are selected? How to align? In what orientation?

25. 6)-c): Steps using command windows cannot filmed. Please do not highlight these steps.

26. 7)-a).2-7)-a).3: Please specify what software is used. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

27. 7)-a).4: Please ensure that all text is written in imperative tense.

28. 7)-a).5: Please ensure that all text is written in imperative tense.

29. 8)-b).1: Please ensure that all text is written in imperative tense.

30. 8)-b).3: Please ensure that all text is written in imperative tense.

31. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

**Dear Editor,**

**We have answered all the points that you and the reviewers raised. Our answers to the reviewers’ comments are in bold below. We hope that the manuscript now meets the criteria to be published in JOVE. We thank you for your help with this manuscript.**

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

This manuscript describes setup consisting of a custom lightsheet microscope and an optical tweezer. The authors describe how to align and couple the optical tweezers to the custom microscope, and how to perform experiments on living specimen to probe mechanical properties, such as intracellular contact, stiffness and tension in Drosophila melanogaster. Furthermore, the authors share both the control and the analysis softwares needed for steering the optical tweezers and for quantifying the measurements which can be obtained by such a setup.

Major Concerns:

The manuscript seems to describe a setup which is tailored to the author's custom lightsheet. However, the technique described is independent of the type of microscope used for imaging. I therefore encourage the authors to put some more effort into generalizing the description of how to couple an optical tweezers to a general microscope.

**It is true and said in the discussion that the optical tweezers setup can be adapted to other microscopes such as an epi-fluorescence or a spinning-disk microscope. The introduction of the IR trapping laser into the microscope depends on the microscope configuration. This mainly requires the possibility to add a dichroic mirror to combine the paths for imaging and optical manipulation. Most microscopy companies propose modular illumination systems, with a two-layer module, which allow this combination. We have added this information in the main text.**

A troubleshooting section is needed: the authors should try and give helpful hints about what to do or which parameters to check in case a user cannot reproduce what is written in the text. As an example, what are the components/settings to be checked if no bead can be stably trapped?

**Thank you for the suggestion. We have added for few steps of the protocol a troubleshooting section. We hope it will help the reader to overcome possible difficult steps within the protocol.**

Minor Concerns:

The description about how to align and couple the optical tweezers optical elements with each other and finally to the microscope is somehow too crude: importantly, a figure reporting the accurate distances and relative positions of all the optical elements is missing. The authors do report the theoretical values for setting up 4f systems (lines 118-119 and 125) but without knowing where the lenses and mirrors need to be placed, the whole procedure might be hard to grasp for the general reader.

**Distances are now provided in the figure 1.**

Some passages in the protocol might not lead to an optimal alignment if performed according to what the authors write: for example, when describing how to setup the tweezing path the authors write to place and align the two galvanometers and the periscopes and to check whether the IR laser reaches the objective (lines 112 onward, points 5 to 17), but omit to say that the mirrors should be powered and set to zero deflection for best results. Optimizing the optical path when the galvanometers are in a random position might result in sub-optimal light coupling.

**This is true, and we modified the protocol point 2.6 and 2.7.**

It is not clear which input/output of the galvanometer need to be connected with the board analog inputs and outputs (line 173). Please clarify which signal pin of the galvanometer mirror should be connected to the board input and which to the output

**Pins of the driver board of the galvanometer can be localized thanks to the Thorlabs manual. We added a note in the protocol.**

The objective used for imaging and trapping is referred to as having an NA of 1.0 (line 94), but Figure 1 reports it as a 100x/1.1 NA. Which one is correct?

**The numerical aperture of the objective is 1.1. We corrected this in the protocol.**

**Reviewer #2:**

Manuscript Summary:

The authors describe a procedure and instrumentation for applying forces using optical tweezers to Drosophila embryos, and imaging the resulting dynamics using light sheet fluorescence microscopy.

The paper is well motivated. The mechanics that govern the development of animals are poorly understood, and methods to probe these systems are few in number, and are often invasive or difficult to implement. The paper is clearly written. Technically, the authors' work is impressive - this is a difficult project, and the system the authors have developed is admirable. However, the paper has significant flaws, described below.

Major Concerns:

1 A central claim of the work is the ability to measure forces noninvasively. The method, however, does not do this, and I do not think the argument the authors provide makes sense. The authors quite nicely move intercellular junctions and observe their motion. To extract a force from this requires knowledge of the stiffness of the junctions, or their optical properties, neither of which are known. The authors calibrate their force measurement using a three-step procedure. First, they track bead displacements to determine the local viscosity; this assumes a Newtonian fluid, and assumes purely thermal motions (i.e. no active motions), neither of which are in general true for the cytoplasm. Second, they determine the trap stiffness using beads. This is fine, but because the stiffness is itself a consequence of the index of refraction of the beads relative to their surroundings, it is valid only for a "free" bead in the cytosol. Third, the authors tug on a cellular contact line and move it, and then move "beads pushed against contact lines," comparing the displacement between the two to determine the trap stiffness for contact lines relative to the stiffness for the beads. However: the fact that the contact line can be moved implies that its local index of refraction is different than that of the cytoplasm, so bringing a bead near the contact line changes the optical properties around the bead, so the trapped-bead-stiffness will not he the same as what was measured in part 2. Overall, I do not believe that the authors are able to absolutely determine forces, and they certainly don't make a clear case that the numbers they extract are "real." I agree that they can provide relative measurements.

**We agree that the determination of the absolute forces is critical and as we state, we can only estimate them through a three-step procedure, that was already described in Bambardekar, Clément et al. The first step utilizes single bead tracking in the cytosol to determine local viscosity. We are aware that our approach is only valid for Newtonian fluid and for a regime dominated by thermal fluctuations. As explained in Bambardekar, Clément et al, bead trajectories were acquired at a frequency (38 Hz) for which active fluctuations are considered to be small compared to thermal ones (Mizuno et al, Science 2007). This assumption has been directly assessed in the cytoplasm of the early Drosophila embryo by microrheology measurements (Wessel et al, Biophys J. 2015: 21, 1899-907). These measurements have also shown that the cytoplasm is viscously dominated.**

**We agree with the reviewer that the refractive index difference at the cell contacts is likely to modify the trap stiffness on the bead. Thus the estimation of the trap stiffness is subject to an experimental error, but, unfortunately, we haven’t found a better strategy of calibration. To clarify this point, we have added a sentence to avoid confusion: “It is important to recognize that the calibration of the trap stiffness, and thus of the forces on cell contacts is subject to experimental uncertainty: it relies on measurements of the trap stiffness on beads, which can be measured only in the cytosol, but not near cell contacts.”**

2 Though the setup is technically challenging and impressive, there is nothing novel about it. The construction of optical tweezers is well described in many places. Light sheet microscopes, though less common, are also well described elsewhere, and the authors' don't discuss the light sheet design much anyway. The authors have put two well-established techniques together. Though this is technically challenging, I do not see what experimental advance the paper provides. Put differently: if I wanted to build an optical trap on a light sheet microscope, I'd look at how to build an optical trap (if I had never built one), and build it onto a light sheet microscope; there's no "conflict" between the two methods that makes this paper necessary. Again, I appreciate the practical challenge of doing this, but I do not see the conceptual challenge.

**We agree that the two parts of the setup, the light sheet microscope and the optical tweezers, are not novel, and we don’t claim they are. Here we describe a setup, which is the combination of two known techniques and a protocol to probe mechanics in the *Drosophila* embryo. We think that this may be useful to the scientific community. We believe that our protocol enters in the category “innovative applications of existing techniques” (JOVE) and that a text protocol and a video demonstration will be useful.**

**Following the referee’s suggestion, we now also refer to existing protocols for optical tweezers (Svoboda, K., & Block, S. M. 1994).**

3 The technical description the authors provide gives lots of details particular to the exact equipment the authors use, but gives almost no discussion of the general principles. Suppose one uses a different Daq card - what are the relevant features to keep in mind? Why a 1070 nm laser? What is QT Creator? Etc.

**We described the setup as we built and used it. Of course, some elements can be changed and we tried to tell it when possible. For example, other NI cards can be used, and we notified in step 3.1 the required numbers of analog outputs and inputs.**

**QtCreator is a Integrated Development Environment, allowing C++ programming and the use of Qt, a widget library. This IDE is needed for people who want to use the code that we provide. We added a note in step 3.10.**

4 The authors focus 200 mW of IR light onto embryos for their trap. This is a lot of power, and they need to demonstrate that this does not alter the local mechanical properties, and does not damage the embryos.

**In Bambarderkar, Clément et al, we monitored the cell response at different IR laser powers. In particular, we showed the linearity of contacts’ deflection for powers ranging from 50 mW to 300 mW. We noted damaging effects for powers larger than 400 mW.  Therefore, we decided to use 200 mW, which induces quite large deflection without damage. Yet, to fully address this point, we would need to compare our measurements with other mechanical non-invasive measurements. Magnetic tweezers may be an alternative to optical tweezers but this would require an extensive study, which goes beyond the scope of the present manuscript.**

**Reviewer #3:**

Manuscript Summary:

the manuscript is a protocol following two research papers (21 and 22). the idea of the protocol should be to make it easier for other scientists to follow and replicate their experimental set-ups. Unfortunately, so much details is left vage that this is not possible.

Major Concerns:

Many parts are describes sloppily without sufficient detail for the user to copy. The authors should carefully go through all aspects making sure that the descriptions are precise, correct and contain the important detailes, incl manufacturing numbers.

**We provide a list of the main optical and mechanical elements that we use with their reference numbers. This list can be found in a separate table, so that the readers can easily find the pieces of equipment to replicate the set-up. The small optical elements that are not listed are standard and have been purchased from Thorlabs; we added a sentence to make this clear.**

A few examples:

1) In the tweezer path setup, it needs to be mentioned that for maximal trap stiffness the IR beam should just over-illuminate the back-focal aperture of the 100x objective. If the beam is small edge rays get lost and the trap will be weaker and if the NA is only 1.1 won't be very strong.

**We thank you the reviewer for this comment; we have now clarified this point by stating: “Note that the IR beam should just overfill the back-focal aperture of the objective lens to maximize the focalization of the IR beam and thus the trap stiffness.”**

2) "1ul of pink beads in glass cuvette, fill with water" is really unscientific, saying nothing about the bead concentration, the material, source and little about the fluorescence loading, what glass covette (the on shown in fig 2), what volume

**We added the reference for fluorescent beads in the materials table and now precise the amount of water required. Note that the concentration of beads is far from being critical.**

3) "turn the acquisition software on" - what software? acquisition of what? I assume it's for the camera, but really needs to be mentioned

**We refer to the QT acquisition software, described in the protocol. It controls the whole microscope including the optical tweezers and the camera. We tried to make it clearer (everytime the software is mentioned) which part of the software is used.**

4) "Adjust the settings of the shutter controller" - what shutter? camera? IR laser?

... there are many, too many of these 'sloppy' description for me to list. They have to be more specific to make this a useful protocol

**The protocol describes the optical setup and mentions the different elements including the shutter and its controller, the camera and the IR laser. Following the reviewer’s suggestion, we also now specify these elements both in the Figure 1 and the table that provides a comprehensive list of the main optical and hardware devices. The video that will be made for JOVE will also specify these elements; we hope that both the text and the video will make this a useful protocol.**

while the abstract and title promise bead free measurements, they protocol only mentions calibration with a bead injected into the cytosol, then ref. 6 is cited and only little detail is given, ref. 21 and 22 seem more important.

More information needs to be given to this process. In the original publication, the authors claim that pushing the beads agains the cell wall, they know the force. However, now that they should the optical trap can directly move the cell wall, it is clear that the optical properties are such they distort the optical trap profile and the force on the bead agains the wall is not same the as the force on the bead in the cytoplasm. also a protein corona will build on the bead affecting the force as well.

**In the original publication (Bambardekar, Clément et al), we already reported the two possibilities of optical manipulation, either using trapped bead to push the cell contacts and or by direct tweezing of the cell contacts. We agree with the reviewer that the refractive index difference at the cell contacts is likely to modify the trap stiffness on the bead. Thus the estimation of the trap stiffness is subject to an experimental error, but, unfortunately, we haven’t found a better strategy of calibration. To clarify this point, we have added a sentence to avoid confusion: “It is important to recognize that the calibration of the trap stiffness, and thus of the forces on cell contacts is subject to experimental uncertainty: it relies on measurements of the trap stiffness on beads, which can be measured only in the cytosol, but not near cell contacts.”**

Minor Concerns:

Large sections are highlighted yellow, not clear why.

several links in the reference do not work as they are two links in a row

**These are the sections that will be used to prepare the script of the movie.**

**We corrected the links.**